

**APPLICATION**  
**FOR**  
**UNITED STATES PATENT**

**TITLE: RET LIGAND (RetL) FOR STIMULATING NEURAL AND  
RENAL GROWTH**

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## **RET LIGAND (RetL) FOR STIMULATING NEURAL AND RENAL GROWTH**

[0001] This application is a divisional of U.S. Patent Application No. 09/187,907, filed November 6, 1998, which was a continuation-in-part of PCT/US97/07726, filed May 7, 1997, which claims priority to U.S. Provisional Application Nos. 60/017,427, filed May 8, 1996; 60/019,300, filed June 7, 1996; 60/021,859, filed July 16, 1996; 60/023,444, filed August 23, 1996; and 60/043,533, filed April 11, 1997.

### **FIELD OF THE INVENTION**

[0002] This invention relates to nucleotide sequences which encode a Ret ligand (RetL), as well as to methods of stimulating neural and renal growth by treating cells and mammalian subjects with RetL DNA or protein.

### **BACKGROUND OF THE INVENTION**

[0003] One of the goals of current research on cell signaling and receptor activation is to enable therapeutic modulation of processes involved in cell growth and survival. Such processes determine outcome in diverse medical conditions, including organ failure, fetal development, and tumor growth, among others. Each of these conditions is of worldwide clinical importance, and has limited efficacious treatment options. It is an object of the invention to provide compositions and methods for promoting regeneration or survival of damaged tissue, as well as for treating disorders involving the aberrant growth and development of tissues.

[0004] Tissue loss or end-stage organ failure affects millions of people worldwide each year and adds substantially to health care costs. Organ or tissue loss is usually treated by transplanting organs from donors, by surgical reconstruction, or with mechanical devices. Each of these remedies has shortcomings. Transplantation is limited by donor shortage, surgical reconstruction can create other long-term problems, and mechanical devices cannot perform all the functions of a single organ, and therefore cannot prevent progressive deterioration. Thus, a real medical need exists for new solutions to these problems.

**[0005]** Protein factors that affect the growth, differentiation and/or survival of cells may be useful in the treatment of disorders of organs which contain responsive cells. Factors or ligands that interact with receptors of the receptor protein tyrosine kinase (RPTK) family are of particular interest in this regard. These receptors are involved in many cellular programs including cell growth and differentiation, and the genesis of many neoplasias. Thus the factors or ligands that interact with these receptors may prove useful in treating disorders of certain organs where the tissue has been damaged. Alternatively, it may be useful to block the interaction of these factors with their receptors in order to block tumor growth.

**[0006]** The Ret proto-oncogene encodes a receptor tyrosine kinase that is expressed during development in a variety of tissues, including the peripheral and central nervous systems and the kidney. The abnormalities present in ret null mice suggest that Ret is critical for the migration and innervation of enteric neurons to the hindgut, and for proliferation and branching of the ureteric bud epithelium during kidney development (Nature 367, 380-383, 1994). The search for a key component of the Ret signaling pathway, the Ret ligand, has been an area of intensive research.

### **SUMMARY OF THE INVENTION**

**[0007]** The invention provides a purified and isolated DNA molecule coding for a RetL, having the nucleotide sequence of any RetL, but specifically including rat retL1 cDNA (SEQ ID NO:1), partial human retL1 cDNA (SEQ ID NO:8), full-length human retL1 cDNA (SEQ ID NO:10), human retL2 cDNA (SEQ ID NO:12), murine retL3 cDNA (SEQ ID NO:16), partial human retL3 cDNA (SEQ ID NO:18) or human retL3 cDNA (SEQ ID NO:20). The invention further provides a RetL protein, with an amino acid sequence comprising that of rat RetL1 (SEQ ID NO:2), partial human RetL1 (SEQ ID NO:9), full-length human RetL1 (SEQ ID NO:11), human RetL2 (SEQ ID NO:13), murine RetL3 (SEQ ID NO:17), partial human RetL3 (SEQ ID NO:19) or human RetL3 (SEQ ID NO:21).

**[0008]** In another embodiment, the invention includes a DNA sequence which encompasses the sequence (partial human retL1 cDNA (SEQ ID NO:8)) of the insert DNA of clone HRL20,

which is ATCC No. 97604, or the sequence of the insert DNA of clone #230-5A-86-17 (rat retL1 cDNA (SEQ ID NO:1)), which is ATCC No. 98047.

**[0009]** In another embodiment of the invention, a purified and isolated DNA molecule for use in securing expression in a prokaryotic or eukaryotic host cell of a polypeptide product has at least a part of the primary structural conformation and the biological activity of RetL; the DNA may be a) a DNA molecule which comprises rat retL1 cDNA, partial human retL1 cDNA, full-length human retL1 cDNA, human retL2 cDNA, murine retL3 cDNA or human retL3 cDNA, or the complementary strand of rat retL1 cDNA, partial human retL1 cDNA, full-length human retL1 cDNA, human retL2 cDNA, murine retL3 cDNA or human retL3 cDNA; b) DNA molecules which hybridize under stringent conditions to the DNA molecules defined in a) or fragments thereof; or c) DNA molecules which, but for the degeneracy of the genetic code, would hybridize to the DNA molecules defined in a) and b). A purified and isolated DNA molecule coding for a polypeptide fragment or variant of a human RetL having the biological activity of a RetL is also within the invention.

**[0010]** Any of the recombinant DNA molecules of the invention may be operably linked to an expression control sequence.

**[0011]** Also included within the invention are vectors and delivery systems which encompass the DNA molecules or constructs defined elsewhere in this specification. The vector may encompass a DNA molecule encoding a RetL or a variant of a RetL.

**[0012]** The invention includes prokaryotic or eukaryotic host cells stably transformed or transfected by a vector comprising a DNA molecule encoding a native or variant RetL.

**[0013]** A purified and isolated human RetL substantially free of other human proteins is specifically within the invention, as is a process for the production of a polypeptide product having part or all of the primary structural conformation and the biological activity of a RetL. Such a process may include the steps of growing, under suitable culture conditions, prokaryotic or eukaryotic host cells transformed or transfected with any DNA molecule of the invention, in a manner allowing expression of such polypeptide product, and recovering a RetL. The

polypeptide product of the expression in a procaryotic or eukaryotic host cell of a DNA is also included.

**[0014]** The invention also includes proteins and protein fragments, variants and derivatives, whether soluble or membrane bound. In selected embodiments, the protein has an amino acid sequence which comprises rat RetL1, partial human RetL1, full-length human RetL1, human RetL2, murine RetL3, or human RetL3, or is a variant of one of these sequences. In other embodiments, the protein is a fusion protein including Ret or a RetL, fused to another molecule or molecular fragment, such as an immunoglobulin, toxin, imageable compound or radionuclide. Also included are chimeric molecules of RetL.

**[0015]** Other embodiments of the invention include specific monoclonal antibodies to a RetL of the invention. Such an antibody may be associated with a toxin, imageable compound or radionuclide. The invention also includes hybridoma cell lines which produce specific antibodies to Ret, including AA.FF9, AA.HE3, AF.E9, BA.B1, BB.B6, AA.GE7, CD.F11, AH.E3, CD.G4, AG.E7, BD.G6 and BH.G8, as well as subclones of these hybridomas, and the antibodies produced by these hybridomas or subclones of these hybridomas.

**[0016]** The invention further includes a method of promoting growth of new tissue, or promoting survival of damaged tissue in a subject, including administering to the subject a therapeutically effective amount of a compound which interacts with cellular Ret and thereby induces autophosphorylation of Ret. The compound may be RetL1, RetL2, or RetL3, a fragment of a full-length RetL, or an antibody which binds to Ret. The compound may be administered concurrently with a therapeutically effective amount of a second compound, such as GDNF, neurturin or a GDNF-related molecule. While tissues of interest for these methods may include any tissue, preferred tissues include renal tissue, neural tissue, heart, stomach, small intestine, spinal cord, or lung. In one embodiment, the RetL is a soluble RetL. The subject of the methods may be human.

**[0017]** In another method of the invention, Ret signal transduction between a first cell expressing a RetL and a second cell is inhibited by contacting the first cell with a soluble Ret protein or with an antibody to the RetL. The soluble Ret protein may be a fusion protein.

**[0018]** The invention also includes a method for targeting a toxin, imageable compound or radionuclide to a cell expressing Ret, encompassing contacting the cell with a RetL fusion protein or an anti-Ret antibody conjugated to a toxin, imageable compound or radionuclide. The RetL can be RetL1, RetL2 or RetL3. In another method, growth of a tumor cell which expresses Ret is suppressed, with a step of the method being contacting the cell with a fusion protein of a RetL and a toxin or radionuclide, or an anti-Ret antibody conjugated to a toxin or radionuclide. The cell may be within a subject, and the protein or the conjugated antibody is administered to the subject.

**[0019]** Also encompassed within the invention is a method for targeting a toxin, imageable compound or radionuclide to a cell expressing a RetL, comprising contacting the cell with a fusion protein comprising Ret and a toxin, imageable compound or radionuclide, or an anti-RetL antibody conjugated to a toxin, imageable compound or radionuclide. Another embodiment includes the method of suppressing growth of a tumor cell which expresses a RetL, comprising contacting the cell with a fusion protein of Ret and a toxin or radionuclide or with an anti-RetL antibody conjugated to a toxin or radionuclide; the cell may be within a subject, and the protein administered to the subject.

**[0020]** The RetL for any of the methods of the invention can be RetL1, RetL2 or RetL3, or a variant or fragment of RetL1, RetL2 or RetL3.

**[0021]** Methods of gene therapy are also within the invention. One embodiment is a method of treating a subject with a disorder of Ret metabolism, comprising administering to the subject a vector comprising a DNA molecule encoding a RetL, as well as a method of promoting growth of new tissue in a subject, comprising administering such a vector to the subject. Another embodiment includes a method of promoting survival of damaged tissue in a subject, one step of the method being administering a therapeutically effective amount of a vector encoding a RetL to the subject.

**BRIEF DESCRIPTION OF THE DRAWINGS**

[0022] FIGURE 1 is a cDNA sequence (SEQ ID NO:1) and deduced amino acid sequence (SEQ ID NO:2) of rat RetL1. The nucleotide sequence extends from base pair 201 through base pair 1700 of SEQ ID NO:1, and contains the entire open reading frame.

[0023] FIGURE 2A is a partial cDNA sequence (SEQ ID NO:8) and deduced amino acid sequence (SEQ ID NO:9) of human RetL1. This sequence is that of the insert of clone HRL20, deposited as ATCC No. 97604.

[0024] FIGURE 2B is a composite full-length DNA sequence (SEQ ID NO:10) and deduced amino acid sequence (SEQ ID NO:11) of human RetL1.

[0025] FIGURE 3A is a comparison of the nucleotide sequence of human RetL1 (upper line of sequence) with that of rat RetL1 sequence (lower line of sequence). Vertical lines between nucleotides show identity at a position, while a dot indicates a gap at that position.

[0026] FIGURE 3B is a comparison of the amino acid sequence of human RetL1 (upper line of sequence) with that of rat RetL1 sequence (lower line of sequence). Vertical lines between corresponding amino acids show identity at a residue, while a dot indicates a conservative substitution at that residue.

[0027] FIGURE 4A is a schematic diagram of a possible role for Ret and RetL in the interaction between a metanephric mesenchyme cell and a ureteric bud cell.

[0028] FIGURE 4B is a schematic diagram of a method of screening transfectants of a cDNA library for clones that express a RetL. The presence of expressed RetL on transfectants is detected by assessing the binding by those transfectants either of Ret/IgG fusion protein or of Ret/alkaline phosphatase fusion protein.

[0029] FIGURE 5 is a schematic diagram showing the construction of the plasmids used to express the rat Ret/IgG fusion protein.

[0030] FIGURE 6 is a schematic diagram showing the construction of the plasmids used to express the human Ret/IgG fusion protein.

[0031] FIGURE 7 is a cDNA sequence (SEQ ID NO:12) and deduced amino acid sequence (SEQ ID NO:13) of human retL2, as found in clone DSW240. The protein reading frame is contained within nucleotides 25 to 1416.

[0032] FIGURE 8 is a comparison of the amino acid sequence of human RetL2 (upper line of sequence) with that of human RetL1 sequence (lower line of sequence). Vertical lines between amino acids show identity at a position, while a dot indicates a gap at that position.

[0033] FIGURE 9 is a cDNA sequence (SEQ ID NO:16) and deduced amino acid sequence (SEQ ID NO:17) of murine RetL3.

[0034] FIGURE 10 is a cDNA sequence (SEQ ID NO:20) and deduced amino acid sequence (SEQ ID NO:21) of human RetL3.

## **DETAILED DESCRIPTION OF THE INVENTION**

### **Sequence Identification Numbers**

[0035] Nucleotide and amino acid sequences referred to in the specification have been given the following sequence identification numbers:

- SEQ ID NO:1 - rat retL1 cDNA
- SEQ ID NO:2 - rat RetL1 aa
- SEQ ID NO:3 - oligomer kid-13
- SEQ ID NO:4 - oligomer kid-14
- SEQ ID NO:5 - oligomer kid-15
- SEQ ID NO:6 - extracellular rat retL1 cDNA
- SEQ ID NO:7 - extracellular rat RetL1 aa
- SEQ ID NO:8 - partial human retL1 cDNA
- SEQ ID NO:9 - partial human RetL1 aa
- SEQ ID NO:10 - human retL1 cDNA
- SEQ ID NO:11 - human RetL1 aa
- SEQ ID NO:12 - human retL2 cDNA
- SEQ ID NO:13 - human RetL2 aa



SEQ ID NO:14 - partial murine retL3 cDNA (EST AA50083)

SEQ ID NO:15 - partial murine RetL3 aa

SEQ ID NO:16 - murine retL3 cDNA

SEQ ID NO:17 - murine RetL3 aa

SEQ ID NO:18 - partial human retL3 cDNA

SEQ ID NO:19 - partial human RetL3 aa

SEQ ID NO:20 - human retL3 cDNA

SEQ ID NO:21 - human retL3 aa

### **Definitions**

**[0036]** As used herein, the term "RetL" means any protein which specifically interacts with the receptor protein Ret, and which when it interacts with Ret triggers Ret dimerization and/or autophosphorylation of the tyrosine kinase domain of Ret. The DNA sequences which code for RetL and for Ret are termed "retL" and "ret", respectively. A ligand may be soluble, or present as a membrane-bound molecule on the same or on a different cell as the Ret molecule for which it is triggering autophosphorylation. In certain uses or interactions with Ret, the ligand may require additional molecules to trigger autophosphorylation. Ligands of the invention include co-receptors or accessory ligand cofactors. Ligands of the invention further include anti-Ret mAbs which act as Ret antagonists, triggering Ret dimerization and autophosphorylation. The ligand may also be modified in various ways, such as incorporated as a portion of a fusion protein, such as with a toxin or radionuclide.

**[0037]** By "alignment of sequences" is meant the positioning of one sequence, either nucleotide or amino acid, with that of another, to allow a comparison of the sequence of relevant portions of one with that of the other. An example of one method of this procedure is given in Needleman et al. (J. Mol. Biol. 48:443-453, 1970). The method may be implemented conveniently by computer programs such as the Align program (DNASTar, Inc.). As will be understood by those skilled in the art, homologous or functionally equivalent sequences include functionally equivalent arrangements of the cysteine residues within the conserved cysteine

skeleton, including amino acid insertions or deletions which alter the linear arrangement of these cysteines, but do not materially impair their relationship in the folded structure of the protein. Therefore, internal gaps and amino acid insertions in the candidate sequence are ignored for purposes of calculating the level of amino acid sequence homology or identity between the candidate and reference sequences. One characteristic frequently used in establishing the homology of proteins is the similarity of the number and location of the cysteine residues between one protein and another.

[0038] By "cloning" is meant the use of *in vitro* recombination techniques to insert a particular gene or other DNA sequence into a vector molecule. In order to successfully clone a desired gene, it is necessary to employ methods for generating DNA fragments, for joining the fragments to vector molecules, for introducing the composite DNA molecule into a host cell in which it can replicate, and for selecting the clone having the target gene from amongst the recipient host cells.

[0039] By "cDNA" is meant complementary or copy DNA produced from an RNA template by the action of RNA-dependent DNA polymerase (reverse transcriptase). Thus a "cDNA clone" means a duplex DNA sequence complementary to an RNA molecule of interest, carried in a cloning vector.

[0040] By "cDNA library" is meant a collection of recombinant DNA molecules containing cDNA inserts which together comprise a representation of the mRNA molecules present in an entire organism or tissue, depending on the source of the RNA templates. Such a cDNA library may be prepared by methods known to those of skill, and described, for example, in Maniatis et al., Molecular Cloning: A Laboratory Manual, *supra*. Generally, RNA is first isolated from the cells of an organism from whose genome it is desired to clone a particular gene. Preferred for the purposes of the present invention are mammalian, and particularly human, cell lines.

Alternatively, RNA may be isolated from a tumor cell, derived from an animal tumor, and preferably from a human tumor. Thus, a library may be prepared from, for example, a human adrenal tumor, but any tumor may be used.

[0041] As used herein, the term "DNA polymorphism" refers to the condition in which two or more different nucleotide sequences can exist at a particular site in DNA.

[0042] "Expression vector" includes vectors which are capable of expressing DNA sequences contained therein, i.e., the coding sequences are operably linked to other sequences capable of effecting their expression. It is implied, although not always explicitly stated, that these expression vectors must be replicable in the host organisms either as episomes or as an integral part of the chromosomal DNA. A useful, but not a necessary, element of an effective expression vector is a marker encoding sequence, which is a sequence encoding a protein which results in a phenotypic property (e.g. tetracycline resistance) of the cells containing the protein which permits those cells to be readily identified. In sum, "expression vector" is given a functional definition, and any DNA sequence which is capable of effecting expression of a specified contained DNA code is included in this term, as it is applied to the specified sequence. Such vectors are frequently in the form of plasmids, so "plasmid" and "expression vector" are often used interchangeably. However, the invention is intended to include such other forms of expression vectors, including phage, which serve equivalent functions and which may from time to time become known in the art.

[0043] Similarly, a "functional derivative" of a gene of any of the proteins of the present invention is meant to include "fragments", "variants", and "analogues" of the gene, which may be "substantially similar" in nucleotide sequence, and which encode a molecule possessing similar activity.

[0044] "GDNF-related molecule" means any molecule which is at least 40% homologous to either GDNF or neurturin, and is also capable of specifically binding a RetL.

[0045] The term "gene" means a polynucleotide sequence encoding a peptide.

[0046] By "homogeneous" is meant, when referring to a peptide or DNA sequence, that the primary molecular structure (i.e., the sequence of amino acids or nucleotides) of substantially all molecules present in the composition under consideration is identical.

[0047] The term "oligonucleotide" as used herein in referring to probes, oligomer fragments to be detected, oligomer controls, unlabeled blocking oligomers and primers for amplification of sequences is defined as a molecule comprised of more than three deoxyribonucleotides or

ribonucleotides. Its exact size will depend on many factors, which in turn depend on the ultimate function or use of the oligonucleotide.

[0048] The term "probe" refers to a ligand of known qualities capable of selectively binding to a target antiligand. As applied to nucleic acids, the term "probe" refers to a strand of nucleic acid having a base sequence complementary to a target strand.

[0049] "Recombinant host cells" refers to cells which have been transformed with vectors constructed using recombinant DNA techniques. As defined herein, the antibody or modification thereof produced by a recombinant host cell is by virtue of this transformation, rather than in such lesser amounts, or more commonly, in such less than detectable amounts, as would be produced by the untransformed host.

[0050] As used herein, the terms "restriction endonucleases" and "restriction enzymes" refer to bacterial enzymes each of which cut double-stranded DNA at or near a specific nucleotide sequence.

[0051] As used herein, the term "restriction fragment length polymorphism" ("RFLP") refers to the differences among individuals in the lengths of a particular restriction fragment.

[0052] A molecule is said to be "substantially similar" to another molecule if the sequence of amino acids in both molecules is substantially the same, and if both molecules possess a similar biological activity. Thus, provided that two molecules possess a similar activity, they are considered variants as that term is used herein even if one of the molecules contains additional amino acid residues not found in the other, or if the sequence of amino acid residues is not identical. As used herein, a molecule is said to be a "chemical derivative" of another molecule when it contains additional chemical moieties not normally a part of the molecule. Such moieties may improve the molecule's solubility, absorption, biological half life, etc. The moieties may alternatively decrease the toxicity of the molecule, eliminate or attenuate any undesirable side effect of the molecule, etc. Moieties capable of mediating such effects are disclosed, for example, in Remington's Pharmaceutical Sciences, 16th ed., Mack Publishing Co., Easton, Penn. (1980).

**[0053]** By "vector" is meant a DNA molecule, derived from a plasmid or bacteriophage, into which fragments of DNA may be inserted or cloned. A vector will contain one or more unique restriction sites, and may be capable of autonomous replication in a defined host or vehicle organism such that the cloned sequence is reproducible.

**[0054]** By "substantially pure" is meant any protein of the present invention, or any gene encoding any such protein, which is essentially free of other proteins or genes, respectively, or of other contaminants with which it might normally be found in nature, and as such exists in a form not found in nature.

### **Compounds of the Invention**

**[0055]** The invention includes cDNA coding for a RetL, such as the nucleotide sequence of rat retL1 cDNA, partial human retL1 cDNA, full-length human retL1 cDNA, human retL2 cDNA, murine retL3 cDNA or human retL3 cDNA. In addition, the compounds of the invention include sequences which include the above sequences, or are derivatives of one of these sequences. The invention also includes vectors, liposomes and other carrier vehicles which encompass one of these sequences or a derivative of one of these sequences. The invention also includes proteins transcribed and translated from rat retL1 cDNA, partial human retL1 cDNA, full-length human retL1 cDNA, human retL2 cDNA, murine retL3 cDNA or human retL3 cDNA, including but not limited to rat RetL1, partial human RetL1, full-length human RetL1, human RetL2, murine RetL3, or human RetL3, and their derivatives and variants.

**[0056]** One embodiment of the invention includes soluble variants of a RetL. Soluble variants lack at least a portion of the intramembrane section of the native RetL. In some examples, the soluble variant lacks the phosphatidylinositol glycan linkage of the native RetL. Soluble variants include fusion proteins which encompass derivatives of RetL that lack a phosphatidylinositol motif.

**[0057]** Variants can differ from naturally occurring RetL in amino acid sequence or in ways that do not involve sequence, or both. Variants in amino acid sequence are produced when one or more amino acids in naturally occurring RetL is substituted with a different natural amino

acid, an amino acid derivative or non-native amino acid. Particularly preferred variants include naturally occurring RetL, or biologically active fragments of naturally occurring RetL, whose sequences differ from the wild type sequence by one or more conservative amino acid substitutions, which typically have minimal influence on the secondary structure and hydrophobic nature of the protein or peptide. Variants may also have sequences which differ by one or more non-conservative amino acid substitutions, deletions or insertions which do not abolish the RetL biological activity. Conservative substitutions typically include the substitution of one amino acid for another with similar characteristics such as substitutions within the following groups: valine, glycine; glycine, alanine; valine, isoleucine; aspartic acid, glutamic acid; asparagine, glutamine; serine, threonine; lysine, arginine; and phenylalanine, tyrosine. The non-polar (hydrophobic) amino acids include alanine, leucine, isoleucine, valine, proline, phenylalanine, tryptophan and methionine. The polar neutral amino acids include glycine, serine, threonine, cysteine, tyrosine, asparagine and glutamine. The positively charged (basic) amino acids include arginine, lysine and histidine. The negatively charged (acidic) amino acids include aspartic acid and glutamic acid.

**[0058]** Other conservative substitutions can be taken from the table below, and yet others are described by Dayhoff in the Atlas of Protein Sequence and Structure (1988).

**TABLE 1: CONSERVATIVE AMINO ACID REPLACEMENTS**

For Amino Acid	Code	Replace with any of
Alanine	A	D-Ala, Gly, beta-Ala, L-Cys, D-Cys
Arginine	R	D-Arg, Lys, homo-Arg, D-homo-Arg, Met, D-Met, Ile, D-Ile, Orn, D-Orn
Asparagine	N	D-Asn, Asp, D-Asp, Glu, D-Glu, Gln, D-Gln
Aspartic Acid	D	D-Asp, D-Asn, Asn, Glu, D-Glu, Gln, D-Gln
Cysteine	C	D-Cys, S-Me-Cys, Met, D-Met, Thr, D-Thr
Glutamine	Q	D-Gln, Asn, D-Asn, Glu, D-Glu, Asp, D-Asp
Glutamic Acid	E	D-Glu, D-Asp, Asp, Asn, D-Asn, Gln, D-Gln
Glycine	G	Ala, D-Ala, Pro, D-Pro, Beta-Ala, Acp
Isoleucine	I	D-Ile, Val, D-Val, Leu, D-Leu, Met, D-Met
Leucine	L	D-Leu, Val, D-Val, Met, D-Met
Lysine	K	D-Lys, Arg, D-Arg, homo-Arg, D-homo-Arg, Met, D-Met, Ile, D-Ile, Orn, D-Orn
Methionine	M	D-Met, S-Me-Cys, Ile, D-Ile, Leu, D-Leu, Val, D-Val, Norleu

Phenylalanine	F	D-Phe, Tyr, D-Thr, L-Dopa, His, D-His, Trp, D-Trp, Trans 3,4 or 5-phenylproline, cis 3,4 or 5 phenylproline
Proline	P	D-Pro, L-I-thioazolidine-4-carboxylic acid, D- or L-1-oxazolidine-4-carboxylic acid
Serine	S	D-Ser, Thr, D-Thr, allo-Thr, Met, D-Met, Met(O), D-Met(O), Val, D-Val
Threonine	T	D-Thr, Ser, D-Ser, allo-Thr, Met, D-Met, Met(O), D-Met(O), Val, D-Val
Tyrosine	Y	D-Tyr, Phe, D-Phe, L-Dopa, His, D-His
Valine	V	D-Val, Leu, D-Leu, Ile, D-Ile, Met, D-Met

**[0059]** Other variants within the invention are those with modifications which increase peptide stability. Such variants may contain, for example, one or more non-peptide bonds (which replace the peptide bonds) in the peptide sequence. Also included are: variants that include residues other than naturally occurring L-amino acids, such as D-amino acids or non-naturally occurring or synthetic amino acids such as beta or gamma amino acids and cyclic variants. Incorporation of D- instead of L-amino acids into the polypeptide may increase its resistance to proteases. See, e.g., U.S. Patent 5,219,990.

**[0060]** The peptides of this invention may also be modified by various changes such as insertions, deletions and substitutions, either conservative or nonconservative where such changes might provide for certain advantages in their use. Splice variants are specifically included in the invention.

**[0061]** In addition to substantially full-length polypeptides, the present invention provides for biologically active fragments of the polypeptides. A RetL polypeptide or fragment is biologically active if it exhibits a biological activity of naturally occurring RetL. Such biological activities



include the ability to specifically bind the extracellular portion of Ret, with an affinity that is at least 50% of, and preferably at least equal to, the affinity of naturally occurring RetL for the extracellular portion of Ret. Another biological activity is the ability to bind to an antibody which is directed at an epitope which is present on naturally occurring RetL.

**[0062]** In other embodiments, variants with amino acid substitutions which are less conservative may also result in desired derivatives, e.g., by causing changes in charge, conformation and other biological properties. Such substitutions would include for example, substitution of hydrophilic residue for a hydrophobic residue, substitution of a cysteine or proline for another residue, substitution of a residue having a small side chain for a residue having a bulky side chain or substitution of a residue having a net positive charge for a residue having a net negative charge. When the result of a given substitution cannot be predicted with certainty, the derivatives may be readily assayed according to the methods disclosed herein to determine the presence or absence of the desired characteristics.

**[0063]** Generally, substitutions that may be expected to induce changes in the functional properties of Ret polypeptides are those in which: (i) a hydrophilic residue, e.g., serine or threonine, is substituted by a hydrophobic residue, e.g., leucine, isoleucine, phenylalanine, or alanine; (ii) a cysteine residue is substituted for (or by) any other residue; (iii) a residue having an electropositive side chain, e.g., lysine, arginine or histidine, is substituted for (or by) a residue having an electronegative charge, e.g., glutamic acid or aspartic acid; or (iv) a residue having a bulky side chain, e.g., phenylalanine, is substituted for (or by) one not having such a side chain, e.g., glycine.

**[0064]** Variants within the scope of the invention include proteins and peptides with amino acid sequences having at least sixty percent homology with rat RetL1 (SEQ ID NO:2), partial human RetL1 (SEQ ID NO:9), full-length human RetL1 (SEQ ID NO:11), human RetL2 (SEQ ID NO:13), murine RetL3 (SEQ ID NO:17), partial human RetL3 (SEQ ID NO:19) or human RetL3 (SEQ ID NO:21). More preferably the sequence homology is at least eighty, at least ninety percent, or at least ninety-five percent. For the purposes of determining homology the length of comparison sequences will generally be at least 8 amino acid residues, usually at least

20 amino acid residues. Variants of the compounds of the invention also includes any protein which 1) has an amino acid sequence which is at least forty percent homologous to a RetL protein of the invention, and also which 2) after being placed in an optimal alignment with the RetL sequence (as depicted for RetL1 and RetL2 in Figure 8), has at least 80% of its cysteine residues aligned with cysteines in the RetL protein of the invention.

[0065] Just as it is possible to replace substituents of the scaffold, it is also possible to substitute functional groups which are bound to the scaffold with groups characterized by similar features. Such modifications do not alter primary sequence. These will initially be conservative, i.e., the replacement group will have approximately the same size, shape, hydrophobicity and charge as the original group. Non-sequence modifications may include, for example, in vivo or in vitro chemical derivatization of portions of naturally occurring RetL, as well as changes in acetylation, methylation, phosphorylation, carboxylation or glycosylation.

[0066] Also included within the invention are agents which specifically bind to a protein of the invention, or a fragment of such a protein. These agents include Ig fusion proteins and antibodies (including single chain, double chain, Fab fragments, and others, whether native, humanized, primatized, or chimeric). Additional descriptions of these categories of agents are in PCT application 95/16709, the specification of which is herein incorporated by reference.

## **EXPERIMENTAL PROCEDURE**

### **Overview of Strategy**

[0067] The general strategy used to clone RetL1 is shown in Figures 4A and 4B. Our strategy was based on the premise that at least a RetL is expressed on the metanephric mesenchyme of the developing kidney as a membrane protein (although it is possible that the ligand is also expressed in a soluble form; Figure 4A). The RetL interacts with the Ret receptor on the ureteric bud cell, activating its tyrosine kinase cytoplasmic domain and sending a signal to the nucleus, which in turn activates genes involved in the growth and branching of the ureteric bud. Therefore, proteins containing the extracellular domain of Ret fused to either the F<sub>c</sub> portion of human immunoglobulin G1 (IgG1) or alkaline phosphatase (AP) can be used as part of a strategy to

clone RetL as shown in Figure 4B. The fusion proteins, the expression libraries and other reagents used in the cloning of RetL1 are described below.

[0068] We first isolate a cDNA for rat RetL1 and then use it as a probe to isolate a cDNA for human RetL1. cDNAs are subsequently isolated for RetL2 and RetL3.

### **Generation of Reagents Required for Direct Expression Cloning of Ret Ligands**

#### **1. Isolation of cDNA Encoding Rat Ret Extracellular Domain**

[0069] To identify RetL1, fusion proteins are generated consisting of the extracellular domains of either rat or human Ret fused to a protein, in one example the human Fc portion of IgG1, and in another example alkaline phosphatase. Both fusion partners can be easily assayed to detect cells which express the ligand as illustrated in Figure 4B.

[0070] Since a cDNA coding for rat Ret has never been disclosed, we isolate a cDNA encoding the extracellular domain of the rat Ret receptor using the Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) method. We compare the two nucleotide sequences for human (Genbank Accession numbers M57464 and X15262) and murine (Genbank Accession number X67812) ret and design oligonucleotide primers from regions of high identity between the two sequences. A sense oligomer called kid-013 (SEQ ID NO:3; contains nucleotides 150-169 of Genbank sequence X15262) is chosen from the 5' end of the human ret cDNA sequence overlapping the ATG initiation codon. It includes nucleotides on its 5' end encoding a Not I restriction site for the purpose of cloning. Two antisense oligomers called kid-014 (SEQ ID NO:4; contains the complement of nucleotides 1819-1839 of Genbank sequence M57464) and kid-015 (SEQ ID NO:5; contains the complement of nucleotides 1894-1914 of Genbank sequence X67812) are chosen, respectively, from the human and murine cDNA sequences immediately 5' to the sequences that encode the transmembrane domains. Oligomers kid-014 and kid-015 contain additional nucleotides at their 5' ends that encode a SalI restriction site for the purpose of cloning.

[0071] Total RNA is isolated from the day 14 embryonic rat kidney and mRNA is purified using oligo-dT chromatography. mRNA is converted to cDNA using AMV reverse transcriptase

and the cDNA is converted to double stranded cDNA and amplified using Taq polymerase in a standard polymerase chain reaction with oligomers kid-013 and kid-015. The synthesis of a 1942 bp PCR fragment is confirmed by running an aliquot of the PCR reaction on a 1% agarose gel. The rest of the PCR fragment is digested with Not1 and Sal1 and cloned into pSAB132 previously digested with Not1 and Sal1. The resulting plasmid is called pJC011. The entire insert of plasmid pJC011 contained between the Not1 and Sal1 sites is sequenced, and is shown as extracellular rat retL1 cDNA, SEQ ID NO:6. A translation of this sequence reveals the peptide sequence (SEQ ID NO:7) for extracellular rat RetL1. Because oligomers for PCR were chosen from human and mouse sequences of ret, it is possible that the nucleotide sequence shown as that of extracellular rat ret cDNA, and the peptide sequence shown as that of extracellular rat Ret, may differ from the natural rat ret nucleotide and Ret peptide sequences in the regions of kid-013 and kid-015 sequences. Subsequently, ret cDNA clones are isolated from a day 18 rat embryonic kidney cDNA library and a few nucleotide changes are observed in the primer regions resulting in two amino acid changes. One change is in the signal sequence (arginine at position 5 to threonine) and one change is near the end of the extracellular domain (glutamic acid at position 633 to alanine). Both of these changes should not affect ligand binding.

## 2. Ret/IgG Fusion Proteins

**[0072]** Fusion proteins are generated consisting of the extracellular domains of the rat (aa residues #1-637) and human (aa residues #1-636) Ret receptors fused to the Fc portion of human IgG1.

**[0073]** The construction of the plasmids used to express the rat Ret/IgG fusion protein is shown schematically in Figure 5. In order to construct a gene encoding the rat Ret/IgG fusion protein, we digest pJC011 (described above) containing the rat Ret extracellular domain with Sal1, and ligate it to a 700 bp Sal1 fragment from plasmid 2-4, to create plasmid pJC012. This Sal1 fragment contains part of the Fc domain of human IgG1 originally derived from plasmid pSAB144. Plasmid 2-4 was created previously via a three way ligation: a Not1 - Sal1 fragment

generated by PCR containing the extracellular domain of the rabbit TGF-beta type II receptor; a 693 bp Sal1 - Not1 fragment from pSAB144 containing part of the Fc domain of human IgG1; and Not1 digested pSAB132. As shown in Figure 5, a fragment containing the Fc domain can be released from the 2-4 plasmid as a 700 bp Sal1 fragment. pJC012 is transfected into COS cells and the rat Ret/IgG fusion protein is purified from the medium 48 hrs later using Protein-A Sepharose chromatography. In order to make a stable cell line producing the rat Ret/IgG protein, the 2612 bp Not1 fragment from pJC012 containing the entire rat Ret/IgG fusion protein is isolated and cloned into the Not1 site of expression vector pMDR901. The resulting plasmid is called pJC022. Plasmid pJC022 is transfected into CHO cells to generate stable cell lines. The highest producing cell line is suspension adapted. Typical yields for the rat Ret/IgG CHO line are 75 mg/L.

**[0074]** The construction of the plasmids used to express the human Ret/IgG fusion protein is shown schematically in Figure 6. In order to construct a gene encoding the human Ret/IgG fusion protein, we obtain a plasmid containing a cDNA encoding the human Ret receptor from Dr. M.Takahashi (Department of Pathology, Nagoya University, School of Medicine, Nagoya, Japan). A PCR fragment is generated from this plasmid using oligomers kid-013 and kid-014. The PCR fragment is treated with Klenow fragment followed by digestion with Not1 to produce a PCR fragment with a sticky Not1 end and one blunt end. This fragment is cloned into the vector pGEM11zf(+) previously digested with EcoR1, treated with Klenow fragment, and digested with Not1, in order to generate a sticky Not1 end and one blunt end. The resulting plasmid is called pJC013. The 1916 bp Not1 - Sal1 fragment from pJC013 is isolated after a complete digestion with Not1 and a partial digestion with Sal1, and ligated to the 693 bp Sal1 - Not1 fragment from pSAB144 containing part of the Fc domain of human IgG1, and the pSAB132 expression vector digested with Not1. The resulting plasmid is called pJC015. The insert in plasmid pJC013 is sequenced and found to contain a single nucleotide difference which changes one amino acid in the extracellular domain of human Ret (Genbank sequence M57464 has a C at position 812, whereas pJC013 has a T at the corresponding position; this results in a change in amino acids from alanine to valine at position 294 of the human Ret protein sequence).

This nucleotide is corrected back to the C residue specified by Genbank sequence M57464 by site specific mutagenesis of plasmid pJC013, producing plasmid pJC023. A 585 bp BstE2 fragment from pJC023 containing the repaired nucleotide sequence is isolated and cloned into plasmid pJC015 from which the 585 bp BstE2 fragment containing the variant nucleotide has been removed. The new plasmid is called pJC024. The 2609 bp Not1 fragment from pJC024 containing the entire human Ret/IgG fusion protein is isolated and cloned into the Not1 site of expression vector pMDR901. The resulting plasmid is called pJC025. Plasmid pJC025 is transfected into CHO cells to generate stable cell lines. The highest producing cell line is suspension adapted. Typical yields for the human Ret/IgG CHO line are 6 mg/L.

[0075] Further details on production of the vectors employed in the methods of the invention are given in PCT applications 94/01456 and 92/02050, the specifications of which are herein incorporated by reference.

### 3. Bioactivity of the Ret/IgG Fusion Proteins

[0076] To determine if the Ret/IgG fusion proteins that we produce are bioactive and therefore would be good screening reagents for the cloning of a RetL, we perform several organ culture assays for bioactivity. The organ culture assay consists of growing day 13-14 embryonic rat kidneys in organ culture for 3-5 days in the presence of the Ret/Ig fusion protein at a concentration of 50 ug/ml. Kidneys are also cultured in the presence of LFA-3TIP/IgG or vehicle buffer. After the culture period, some of the kidneys are stained with the fluorescent lectin Dolichos Biflorus Agglutinin (DB lectin) which stains the collecting duct tissues, which are epithelial cells derived from the ureteric bud. These "DB" positive cells mark the Ret-positive cells, since Ret is expressed in the ureteric bud and its epithelial derivatives. This provides a gross assessment of the Ret/IgG fusion protein on the growth and development of the embryonic kidney. There is a clear difference in collecting duct morphology and growth between kidneys which have been cultured with LFA-3TIP and those cultured with the rat Ret/IgG fusion protein.. The Ret/IgG-treated kidneys have collecting ducts which show significantly less branching and are typically smaller overall.

[0077] Paraffin sections are prepared from other kidneys for histological examination. Embryonic kidneys are treated with control buffer or with Ret/IgG, then stained with hematoxylin and eosin. The Ret/Ig-treated embryonic kidney exhibits less branching of the collecting ducts than the control buffer-treated embryonic kidneys. In addition, Ret/IgG-treated kidneys have fewer tubules. We have also observed this effect with the human Ret/IgG fusion protein. These observations are consistent with the fusion proteins blocking the inductive signal between the mesenchyme and the ureteric bud. Therefore we conclude that the fusion protein is a good reagent for cloning a RetL.

#### 4. Ret/alkaline phosphatase Fusion Protein

[0078] Receptor/alkaline phosphatase (AP) fusion proteins have been used successfully to identify and clone ligands for c-kit (Cell 63:185, 1990), ligands for members of the eph family of orphan receptors (Cell 79:157, 1994), and recently to clone a receptor for leptin, the product of the ob gene (Cell 83:1263, 1995). Plasmids encoding the rat Ret/AP fusion protein are constructed and the rat Ret/AP protein is produced in COS7 cells in cell factories. Subsequently, a stable NIH3T3 cell line is generated expressing on average 10 mg/L of fusion protein. SDS-PAGE analysis of the rat Ret/AP protein indicates that its size is consistent with the predicted molecular weight, and gel filtration analysis indicates that it is produced as a dimer. Partial purification is achieved by affinity chromatography on an anti-AP column.

#### 5. Anti-Ret Antibodies

[0079] A rabbit polyclonal antibody is generated against the rat Ret/IgG fusion protein. The antibody works on Western blots, FACS analysis of Ret positive cell lines, and immunohistochemistry of embryonic kidney sections.

[0080] A panel of hamster anti-rat Ret monoclonal antibodies is generated. Rat Ret/IgG fusion protein, coupled to Protein A Sepharose, is used to immunize Armenian hamsters. 316 clones are obtained after the fusion and screened for their ability to bind rat Ret fusion proteins and/or human IgG in an ELISA assay. 11 clones produce antibodies that bind only to rat

Ret/IgG (and rat Ret/AP), but not human IgG. The cross reactivity to human Ret is assayed by FACS; four clones produce antibodies that can bind to the Ret positive human cell line THP-1. The following table summarizes the Ret binding properties of twelve monoclonal antibodies.

Clone	ELISA rat Ret/Ig	FACS human THP-1
AA.FF9.5	+	-
AA.HE3.7	+	+
AF.E9.5	+	-
BA.B1.16	+	-
BB.B6	+	-
AA.GE7.3	+	-
CD.F11.2	+	-
AH.E3.11	+	+
CD.G4.2	+	+
AG.E7.9	+	-
BD.G6	+	+
BH.G8	-	-



## 6. cDNA Expression Libraries

[0081] We prepare cDNA libraries from rat embryonic kidney, one in the CDM8 vector which utilizes the SV40 origin for amplification, and one in a modified InVitrogen vector, pCEP4, which utilizes the EBV origin for amplification. This modified vector, CH269, has the EBNA-1 gene sequence removed. The EBNA-1 protein interacts with the EBV origin, but the gene is not needed on the vector when cells are used which stably express the EBNA protein. The library in the CDM8 vector contains  $1.5 \times 10^6$  clones with an average insert size of 1.18 kb, while the library in the CH269 vector contains approximately  $1 \times 10^6$  clones with an average insert size of 1.5 kb.

### **Expression Cloning of Ret Ligand RetL1**

#### **A. Cloning of Rat Ret Ligand RetL1**

##### **1. Initial Attempts at Expression Cloning of Ret Ligand RetL1 -**

[0082] A number of direct expression methods have been tried to clone RetL1. All of these methods are based on the concept illustrated in Figure 4B. cDNAs from a cDNA library are introduced into mammalian cells; cells that receive RetL1 can be identified using the Ret fusion proteins. Although the three approaches described below were unsuccessful, important knowledge and expertise was acquired, which was deployed in a subsequent approach that met with success.

[0083] a. Panning Method with Ret/IgG - The rat Ret/IgG fusion protein is used in an attempt to isolate RetL1 by direct expression cloning using a panning method (Aruffo and Seed, Proc. Natl. Acad. Sci. 84: 8753-8757 (1987)). A day 18 embryonic rat kidney cDNA library in CDM8 is used for the panning effort. Pools of cDNAs from this library (5,000 - 10,000 cDNAs per pool) are introduced into COS cells using the DEAE-dextran method. After 48 hours, the cells are removed from the plates with EDTA, incubated with the fusion protein, and subsequently panned on plates coated with anti-human IgG<sub>1</sub> antibody. DNA is recovered from cells that adhered, transformed back into *E. coli*, and subsequently isolated for a second round of panning.

We are unable to see any cells bind after the third round of panning, and very few clones are obtained after transformation of the Hirt DNA back into *E. Coli*. A VCAM cDNA, used in conjunction with an anti-VCAM monoclonal antibody as a positive control, could only be diluted to a ratio of 1:100 and still be detected, indicating that our pool sizes are probably too large. Analysis of some of the clones that are obtained after the second round of panning, indicates that the clones are undergoing rearrangement and deletion.

**[0084]** b. Preparative FACS Method with Ret/IgG - 80,000 cDNA clones from the day 18 embryonic rat kidney library (CDM8 vector) are introduced into COS7 cells and subjected to preparative FACS using the rat Ret/IgG protein followed by a fluor-tagged secondary antibody. The top 0.5% and 0.9% of fluorescing cells are collected and the plasmid DNA is recovered by Hirt lysis. The DNA is electroporated back into *E. coli*: 228 clones are obtained for the 0.5% pool and 752 clones for the 0.9% pool. DNA is recovered from the bacterial clones and a second round of preparative FACS is performed. Plasmids recovered from bacterial clones at the end of the second round are analyzed and found to contain large deletions and rearrangements.

**[0085]** c. Colorimetric Detection Method with Ret/AP - COS cells are transfected with 400 pools of the cDNA clones (1000 clones per pool) from the day 18 rat embryonic kidney cDNA library (CDM8 vector) and stained with the Ret/AP protein and a colorimetric substrate for alkaline phosphatase. The transfected cells are inspected under a microscope for positive signals. In one experiment, five potential positives were re-analyzed, but all were negative.

**[0086]** As a control for the Ret/AP protein, a VCAM/AP protein is produced by fusing the first two domains of human VCAM to the N-terminus of placental AP. (VCAM binds to the integrin VLA4, which is composed of two chains, alpha-4 and beta-1). Transient transfections of COS cells produces sufficient VCAM/AP protein for control experiments. The VCAM/AP protein is compared to VCAM/IgG directly coupled to AP, and to VCAM/IgG plus an AP coupled secondary antibody, in order to assess their ability to detect VLA4 on COS cells transfected with the alpha-4 chain cDNA (COS cells already express the beta-1 chain). The

results show that while the VCAM/AP protein could detect VLA4 on transfected cells, the best detection is afforded by the VCAM/IgG protein in combination with an AP coupled secondary antibody.

**[0087]** d. Methodological Conclusions:

Three major conclusions emerged from these initial cloning efforts:

**[0088]** 1) Methods which require that plasmid DNA be recovered for subsequent rounds (i.e., panning and preparative FACS) are not suitable when the abundance of the target cDNA is low, because of rearrangements and deletions that occur during these subsequent rounds. Based on the low expression of Ret, there is good reason to suspect that the expression of RetL1 is also low. The preferred approach is to transfect in pools and use a detection method that allows a positive pool to be identified. The original pool can then be broken down, with no need to recover the transiently expressed DNA from transfected cells.

**[0089]** 2) The Ret/IgG protein when coupled to a secondary reagent affords better detection capability than the Ret/AP protein.

**[0090]** 3) Control experiments with a VCAM/IgG control protein (and an AP coupled secondary antibody) and the alpha-4 integrin cDNA (diluted into CDM8 vector and transfected into COS cells) indicate that our detection capability is just about one in a thousand (i.e., the pool size cannot exceed 1000 clones). To attain an improved level of sensitivity, we changed from an SV40 origin based vector (expressed in COS cells) to an EBV origin based vector (expressed in EBNA positive cell lines). EBV origin based vectors are maintained as episomes and are not as toxic to the cell as the SV40 origin based vectors after amplification. Considerable evidence exists that genes can be expressed at higher levels in these vectors and that cDNAs can be diluted much further (i.e., up to 1 to 80,000) and still be detected.

2. Screening of Pools from the EBV Origin Based cDNA library

**[0091]** We screen pools of clones from the day 18 rat embryonic kidney cDNA library (CH269 vector with the EBV origin) with the rat Ret/IgG fusion protein. In one experiment, 256 pools,

each containing 5000 clones from the library, are generated. Briefly, an aliquot of the cDNA library is titered, 5000 cells are plated (256 times), and are allowed to grow overnight. The colonies are scraped into medium: part of the culture is used to generate a glycerol stock for the pool (stored at -70) and part is used for a plasmid preparation. DNAs from the 256 pools are individually transfected into 293/EBNA cells ( $8 \times 10^5$  on a 60 mm plate) using the lipofection method. After 48 hr, the cells are washed two times with HBHA buffer (0.5 mg/ml BSA, 0.1%  $\text{NaN}_3$ , 20 mM HEPES (pH 7.0)) and incubated with 20 ug/ml rat Ret/IgG in Tris-buffered saline plus 1 mM  $\text{MgCl}_2$  and  $\text{CaCl}_2$  for 60-90 min at RT. Following this incubation, the cells are washed four times with HBHA buffer and then fixed with 60% acetone/3% formaldehyde/ 20 mM HEPES (pH 7.0) for 30 sec. Following two washes with HBS buffer (150 mM NaCl, 20 mM HEPES (pH 7.0)), the cells are incubated with an AP-coupled secondary antibody (goat anti-human IgG Fc-gamma-specific  $\text{F(ab=)}_2$  (Jackson Immuno Research Laboratories; catalog # 109-056-098; 1:5000 dilution in Tris-buffered saline plus 1 mM  $\text{MgCl}_2$  and  $\text{CaCl}_2$ ) for 60 min at RT. The cells are then washed twice with HBS buffer and twice with AP substrate buffer (100 mM Tris-HCl (pH 9.5), 100 mM NaCl, 5 mM  $\text{MgCl}_2$ ) containing 2X Pierce Immuno Pure<sup>R</sup> Phosphatase suppressor (catalog #35002). The last wash is left for 15 min. The AP substrates NBT (0.33 mg/ml) and BCIP (0.17 mg/ml) are then added in AP substrate buffer containing the Pierce AP inhibitor and incubated with the cells for 5-20 min. The plates are then washed twice with water. The plates are then inspected under a dissecting microscope for the presence of purple stained cells.

**[0092]** From an analysis of the 256 pools, 17 positive pools are identified in the primary screen. DNA from each positive pool is re-transfected into 293/EBNA cells and the above procedure repeated along with some additional control experiments to confirm that the staining observed is Ret/IgG specific. 10 out of the 17 positive pools only show staining with Ret/IgG fusion protein and not with another IgG fusion protein.

### 3. Breakdown of Pool #230

**[0093]** As an example, one of the above-described positive pools, designated #230, is broken down into smaller subpools in order to identify the cDNA within the pool that is conferring binding to the Ret/IgG fusion protein. 600 cells from the glycerol stock for pool #230 are plated (10 times) and grown overnight. Colonies on these plates are scraped into medium: one tenth of the culture is used to generate a glycerol stock and the remaining portion is used for a DNA preparation. The ten subpools of 600 clones are designated 230-1A through 230-5A and 230-1B through 230-5B. DNAs from these subpools are transfected into 293/EBNA cells and the procedure described above for staining with the Ret/IgG fusion protein is repeated. One subpool #230-5A is positive for staining with the Ret/IgG protein.

**[0094]** Pool #230-5A is further broken down in order to identify the cDNA with this subpool that is conferring binding to the Ret/IgG fusion protein. Cells from the glycerol stock of pool 230-5A are plated and grown overnight. Colonies are picked into the wells of seven 96-well Bioblocks® and grown overnight. From each 96-well Bioblock, 4 pools of 20 clones and 1 pool of 16 clones are made. Thus 35 pools are generated from the seven Bioblocks® designated 230-5A-71 through 230-5A-105. DNAs are prepared from each of these pools and transfected into 293/EBNA cells and re-assayed with the Ret/IgG fusion protein as described above. Pool #230-5A-86 is positive.

**[0095]** Pool #230-5A-86 is broken down by going back to the Bioblock and identifying the 20 clones that were mixed together to make this pool. DNAs are made from all twenty clones and transfected individually into 293/EBNA cells and re-assayed for Ret/IgG as described above. Pool #230-5A-86-17 is found to be positive.

### 4. Characterization of Clone #230-5A-86-17

**[0096]** Clone #230-5A-86-17 (called retL-17 or clone 17 and deposited as ATCC 98047) is further analyzed by DNA sequencing. The entire nucleotide sequence of the insert of this clone is SEQ ID NO:1 (rat retL1 cDNA), and part of the nucleotide sequence is shown in Figure 1. Within this nucleotide sequence, we find a reading frame coding for a protein of 468 amino acids

(rat RetL1). The predicted protein has a signal sequence with a predicted cleavage after amino acid 24 (Von Heijne et al., Nucl. Acid Res. 14:14683, 1986). The hydrophobic C-terminus indicates that the protein may be linked to the cell via a phosphatidylinositol glycan linkage. There are three predicted N-linked glycosylation sites. These properties are consistent with those expected for a ligand for Ret.

[0097] We can express soluble forms of the rat RetL1 protein by truncating the gene prior to the hydrophobic C terminus. For example, this could be done by truncating after Lysine 435 (rat RetL1). Truncation upstream of this amino acid should also result in the expression of a soluble form of the rat RetL1 protein. The soluble rat RetL1 protein can be expressed by itself or as a part of a fusion with human immunoglobulin, a histidine tag, or a small epitope that is recognized by an antibody.

#### **B. Cloning of Human Ret Ligand RetL1**

[0098] A human embryonic kidney cDNA library in the vector lambda gt10 is purchased from Clontech (catalog #HL5004A). One million plaque forming units from the phage stock are plated on 10 Nunc<sup>TM</sup> plates. Duplicate plaque lifts are made on Schleicher and Schuell Optitran<sup>TM</sup> filters.

[0099] A probe is generated by digesting plasmid rat RetL1 with the restriction enzyme PvuII, followed by agarose gel isolation of a 1.34 kb fragment which corresponds to nt 242-1582 of the rat RetL nucleotide sequence (rat retL1 cDNA). This coding region probe is P<sup>32</sup> labeled by random priming (Feinberg and Vogelstein, Anal. Biochem. 137:266-267, 1984). The filters are hybridized overnight in 300 ml plaque screen PSB buffer (50 mM Tris pH 7.5, 1M NaCl, 0.1% sodium pyrophosphate, 0.2% PVP, and 0.2% Ficoll) containing 10% dextran sulphate, 100 ug/ml tRNA, and 6.7 X 10<sup>7</sup> CPM of the rat probe, at 55C. They are washed twice with plaque screen buffer and twice with 2XSSC/1%SDS at 55C and exposed to film at -70C with an intensifying screen.

[0100] Duplicate positives are cored from the master plates into SM (100 mM NaCl, 10 mM SO<sub>4</sub>, 50 mM Tris pH 7.5) plus gelatin. 24 of these positives are plaque purified. Lambda

miniprep DNA from the purified candidate plaques is digested with Not1, electrophoresed on 1% agarose gel and Southern blotted. The Southern blot is hybridized with the rat RetL1 coding region probe. Clone HRL20 has the longest insert (4.4 kb) which hybridizes intensely to the rat probe. DNA sequence (partial human retL1 cDNA; SEQ ID NO: 8; Figure 2A) and deduced peptide sequence (partial human RetL1; SEQ ID NO: 9; Figure 2A) have been obtained from this clone, confirming that it is the human homologue. This clone encodes most of the coding region, including the 3' end of the coding region.

**[0101]** To obtain the 5' end of the human cDNA, a human fetal kidney Marathon-Ready™ cDNA kit is purchased from Clontech (catalog #7423-1). Antisense oligonucleotides Kid-155, corresponding to the complement of nucleotides 62-81 of SEQ ID NO:8 (partial human retL1 cDNA) and Kid-154, corresponding to the complement of nucleotides 17-43 of SEQ ID NO:8 (partial human retL1 cDNA) are synthesized. PCR is performed using the Advantage™ cDNA PCR kit (Clontech catalog #8417-1) combined with Marathon™ cDNA reagents and the oligonucleotides Kid-155 or Kid-154. The first PCR reaction is set up as follows: 35.5 ul H<sub>2</sub>O; 5.0 ul 10X KlenTaq Buffer; 1.0 ul 10mM dNTP mix; 1.0 ul 50X Advantage™ KlenTaq Polymerase mix. These reagents are combined and mixed. Then 5.0 ul Marathon-Ready™ Fetal Kidney cDNA; 1.0 ul 10uM AP1 primer and 1.5 ul 6.4uM Kid-155 are added (final volume = 50 ul). PCR is carried out in a Perkin-Elmer Cetus DNA Thermal Cycler 480 with the following cycle conditions: 1 cycle of 94C for 1 min; 30 cycles of 94C for 30 sec, 55C for 30 sec, 68C for 4 min. A nested PCR is performed using the product of the first PCR reaction. First, 5 ul of PCR product #1 is diluted 50 fold with TE (final volume 250 ul). The nested PCR reaction contains 35.5 ul H<sub>2</sub>O; 5.0 ul 10X KlenTaq Buffer; 1.0 ul 10mM dNTP mix; 1.0 ul 50X Advantage™ KlenTaq Polymerase mix. These reagents are mixed as above. 5.0 ul diluted PCR product #1; 1.0 ul 10uM AP2 primer and 1.5 ul 6.9uM Kid-154 are then added. Cycle conditions are the same as above. The resultant product of approximately 700 bp is purified on a 1% low-melt agarose gel and phenol extracted. The purified DNA is cloned into the EcoR5 site of pZerO™ (Invitrogen catalog #K2510-01). Sequence information is obtained from multiple isolates, including clones called HRL7G6 and HRL7G8.

**[0102]** The sequence obtained from clone HRL7G8 is found to overlap with the sequence of clone HRL20 (partial human retL1 cDNA) and is used to generate a full-length sequence of human RetL1 (full-length human retL1 cDNA), also shown in Figure 2B. The nucleotide sequence obtained from clone HRL7G8 represents nucleotides 1 to 502 of full-length human retL1 cDNA; the nucleotide sequence from clone HRL20 represents nucleotides 460 to 1682 of full-length human retL1 cDNA. The sequence from clone HRL7G8 is confirmed by sequencing another cDNA clone (GJ102) isolated from the human embryonic kidney lambda gt10 cDNA library described above, using a probe derived from clone HRL7G6. Nucleotides 118 to 1497 comprise the protein reading frame of full-length human retL1 cDNA.

**[0103]** The complete amino acid sequence of human RetL1 is also shown in Figure 2B. As shown by the BESTFIT analysis depicted in Figure 3A, the human retL1 cDNA is 88.2% identical to the rat retL1 cDNA. The peptide comparison (Figure 3B) shows the human putative peptide sequence to be 93.3% identical, and 97.2% similar, to that of the rat.

## **Cloning of Ret Ligand RetL2**

### **A. Cloning of Human RetL2**

**[0104]** The peptide sequence of rat RetL1 (rat RetL1) is used to search the GenBank database with the program BLAST in order to identify related proteins (i.e. isologs). BLAST, or Basic Local Alignment Search Tool, uses the method of Altschul et al. (J. Mol. Biol. 215:403-410, 1990) to search for similarities between a query sequence and all the sequences in the sequence database. The query sequence and the database to be searched can be either peptide or nucleotide in any combination. When the rat RetL1 peptide sequence is queried against the Expressed Sequence Tag (EST) nucleotide database, two significant matches are obtained. One is with GenBank Accession # R02249, a 229 bp EST from a combined human fetal liver and spleen cDNA library, and the other is with Genbank Accession # H12981, a 521 bp EST from a human infant brain cDNA library. The two ESTs share 99% identity in a region of overlap indicating that they are from the same cDNA. Oligonucleotides are generated from the H12981 EST:



KID-228 (GAA TGA CAA CTG CAA GAA GCT GCG CTC CTC; corresponding to nucleotides 38-67 and also to nucleotides 534-563 of SEQ ID NO:12), and antisense oligonucleotide KID-229 (GTG TAC TCG CTG GGC ACC CG; corresponding to the complement of nucleotides 156-175 and also to the complement of nucleotides 652-671 of SEQ ID NO:12).

**[0105]**  $1 \times 10^6$  plaque forming units from a Clontech Human Fetal Liver 5' -Stretch Plus lambda GT10 cDNA library (cat# HL5003a) are screened in duplicate on OPTITRAN™ filters. The filters are hybridized with  $^{32}\text{P}$ -labeled oligonucleotides KID-228 and KID-229 in 400 mls plaque screen buffer (50 mM Tris pH 7.5, 1 M NaCl, 0.1% sodium pyrophosphate, 0.2% Polyvinylpyrrolidine and 0.2% Ficoll) containing 10% Dextran sulfate and 100 ug/ml tRNA and 80 pmole each  $^{32}\text{P}$ -labeled oligonucleotide at 65C overnight. They are washed twice with 2X SSC/1% SDS and twice with 1X SSC/1% SDS and exposed to film. 11 duplicate positives are purified. DNA from each of these clones is analyzed by restriction enzyme digest followed by agarose gel electrophoresis and Southern blotting. The filters are hybridized to KID-228 and KID-229 to confirm that the inserts hybridize to the probe. The insert of clone DSW240 is completely sequenced (human retL2 cDNA, SEQ ID NO:12) and is shown in Figure 7.

**[0106]** Nucleotides 25-1416 comprise the protein reading frame of human retL2 cDNA, which encodes a protein of 464 amino acids (human RetL2; SEQ ID NO:13), and is shown in Figure 7. As shown by the BESTFIT analysis depicted in Figure 8, the human RetL2 protein is 49.1% identical and 63.7% similar to the human RetL1 protein. It shares in common with human RetL1 a hydrophobic N-terminus indicative of a signal sequence and a hydrophobic C-terminus indicative of a phosphatidylinositol glycan linkage motif. In addition, 30 cysteines out of the 31 that are present in each protein are conserved.

#### **B. Demonstration that RetL2 is a Ligand for Ret**

**[0107]** We demonstrate that RetL2 is a ligand for Ret by transfecting 293/EBNA cells with an expression plasmid that contains the insert of clone DSW240 and by showing that the cells can bind a soluble Ret/IgG fusion protein.

**[0108]** The insert of DSW240 is removed using NotI and cloned into the expression vector CH269 which contains an EBV origin and allows for high expression in EBNA positive cell lines. Restriction digests are performed to identify clones that have the correct orientation. Plasmid DNA is prepared from a clone having the correct orientation.

**[0109]** Plasmid DNAs (the retL2 expression plasmid, a retL1 expression plasmid for a positive control, and an expression plasmid containing an unrelated protein for a negative control) are transfected into 293/EBNA cells ( $8 \times 10^5$  on a 60 mm plate) using the lipofection method. After 48 hr, the cells are washed two times with HBHA buffer (0.5 mg/ml BSA, 0.1%  $\text{NaN}_3$ , 20 mM HEPES (pH 7.0)) and incubated with 20 ug/ml rat Ret/IgG in Tris-buffered saline plus 1 mM  $\text{MgCl}_2$  and  $\text{CaCl}_2$  for 60-90 min at room temperature. Following this incubation, the cells are washed four times with HBHA buffer and then fixed with 60% acetone/3% formaldehyde/ 20 mM HEPES (pH 7.0) for 30 sec. Following two washes with HBS buffer (150 mM NaCl, 20 mM HEPES (pH 7.0)), the cells are incubated with an AP-coupled secondary antibody (goat anti-human IgG Fc-gamma-specific  $\text{F(ab')}_2$  (Jackson Immuno Research Laboratories; catalog # 109-056-098; 1:5000 dilution in Tris-buffered saline plus 1 mM  $\text{MgCl}_2$  and  $\text{CaCl}_2$ ) for 60 min at RT. The cells are then washed twice with HBS buffer and twice with AP substrate buffer (100 mM Tris-HCl (pH 9.5), 100 mM NaCl, 5 mM  $\text{MgCl}_2$ ) containing 2X Pierce Immuno Pure<sup>®</sup> Phosphatase suppressor (catalog #35002). The last wash is left for 15 min. The AP substrates NBT (0.33 mg/ml) and BCIP (0.17 mg/ml) are then added in AP substrate buffer containing the Pierce AP inhibitor and incubated with the cells for 5-20 min. The plates are then washed twice with water. The plates are then inspected under a dissecting microscope for the presence of purple stained cells. The presence of purple stained cells indicates that the Ret/fusion protein has bound to the cells and that the retL2 protein is a ligand for Ret. Purple stained cells are also observed after trasfection with the retL1 expression vector but not with the negative control vector.

### **Cloning of Ret Ligand RetL3**

#### **A. Murine RetL3**

[0110] A search of the EST data base with rat RetL1 amino acid sequence discloses two murine ESTs with homology to Ret ligands. These ESTs are AA049894, and AA050083 (which is partial murine retL3 cDNA, SEQ ID NO:14). Plasmids encoding these ESTs are obtained from Genome Systems Inc. (Catalog #475791 and #475497) as bacterial stabs. Plasmid DNA is prepared from single colonies obtained by streaking the stabs onto LB Amp plates. The inserts from these plasmids are sequenced in their entirety. Comparison of the two sequences demonstrates that AA049894, which has a 1.4 kb insert, is contained within AA050083, which has a 1.9 kb insert. Translation of the DNA sequence from AA050083 indicates there is a continuous open reading frame from NT205 to NT1242 (partial murine RetL3; SEQ ID NO:15). This ORF had 37.5% identity to that of rat retL1 and 40.2% identity to rat retL2. However, the open reading frame does not encode a Met or a signal sequence at the 5' end. We examine the 5' ORFs upstream of this region and find a Met in the context of a Kozak consensus sequence for translation initiation and a potential signal sequence for surface expression/secretion. This ORF is out of frame with the downstream ORF indicating that EST AA050083 contains a potential mutation, such as an insertion, deletion, intron or cloning artifact, at its 5' end.

[0111] In order to obtain the correct 5' end, we employ Marathon RACE. Mouse 11-day embryo Marathon-Ready™ cDNA (cat. #7458-1) and an Advantage™ Kit (cat. # 8417-1 ) is purchased from Clontech. Antisense oligonucleotides, Kid-366, corresponding to the complement of nucleotides 847-866 of SEQ ID NO:14 and Kid-365, corresponding to the complement of nucleotides 589-615 of SEQ ID NO:14 are synthesized. PCR is performed using and an Advantage™ cDNA PCR kit (Clontech cat. #8417-1) combined with Marathon™ cDNA reagents and oligonucleotide Kid-366. The first PCR reaction is set up as follows: 35.3 ul H<sub>2</sub>O; 5.0 ul 10X KlenTaq Buffer; 1.0ul 10mM dNTP mix; 1.0 ul 50X Advantage™ KlenTaq Polymerase mix. These reagents are combined and mixed. Then 5.0 ul Marathon-Ready™ mouse 11-day embryo cDNA; 1.0 ul 10uM AP1 primer and 1.7 ul 5.88 uM Kid-366 are added (final volume = 50 ul). PCR is carried out in a Perkin-Elmer Cetus DNA Thermal Cycler 480

with the following cycle conditions: 1 cycle of 94C for 1 min; 5 cycles of 94C for 30 sec, 72C for 4 min; 5 cycles of 94C for 30 sec, 70C for 4 min; 25 cycles 94C for 30 sec, 68C for 4 min. A nested PCR is performed using the product of the first PCR reaction. First, 5 ul of PCR product #1 is diluted 50 fold with TE (Final volume 250 ul). The nested PCR reaction contains 35.5 ul H<sub>2</sub>O; 5.0 ul 10X KlenTaq Buffer; 1.0 ul 10mM dNTP mix; 1.0 ul 50X Advantage<sup>TM</sup> KlenTaq Polymerase mix. These reagents are mixed as above. 5.0 ul diluted PCR product #1; 1.0 ul 10 uM AP2 primer and 3.6 ul 2.8 uM Kid-365 are then added. Cycle conditions are the same as above. The resultant product of approximately 665 bp is purified on a 1% low-melt agarose gel and Qiaex II (Qiagen cat#20021) extracted. The purified DNA is cloned into pNoTA/T7<sup>TM</sup> using PRIME PCR CLONER<sup>TM</sup> cloning system (5 Prime-> 3 Prime cat. #1-725029). Sequence information is obtained from multiple isolates, including clones called DSW252 and DSW253.

[0112] The sequence of DSW252 is found to overlap with SEQ ID NO:14 except that an additional T is present between NT 252 and NT 253 of the SEQ ID NO:14 sequence. This T is also present in the other isolates DSW251 and DSW253. Insertion of this additional base corrects the ORF such that a single 1191 bp ORF (counting from the first Met) encoding 397 amino acids is obtained. This ORF encodes a Met in the context of a canonical translation initiation consensus sequence (Kozak) and includes a signal sequence for surface expression/secretion.

[0113] To obtain a full-length murine clone which is capable of being expressed, a 630 bp Not1-BamH1 fragment of DSW252 and a 1308 bp BamH1-Not1 fragment of AA050083 is purified and ligated to Not 1 digested expression vector CH269. The ligation is transformed into *E. coli* XL1-Blue (Stratagene cat. #200236). Qiawell Ultra minipreps are performed on resultant transformants. These are analysed by restriction digest and gel electrophoresis for correct size and for orientation. This construct is called DSW254. The insert of DSW254 is sequenced in its entirety (murine retL3; SEQ ID NO:16) and the ORF is confirmed as encoding a protein of 397 amino acids (murine RetL3; SEQ ID NO:17). These sequences are also shown in Figure 9. The C-terminus of RetL3 is hydrophobic and indicative of a phosphatidylinositol glycan linkage motif.

## **B. Human RetL3**

[0114] In order to find a candidate tissue source for cloning human RetL3, we utilize northern blots of mouse tissues to determine the expression pattern of murine RetL3. Of the tissues surveyed, expression of RetL3 is highest in heart tissue. A human adult heart cDNA library in the vector lambda gt10 is purchased from Clontech (catalog #HL3026a). One million plaque forming units from the phage stock are plated on 10 Nunc plates. Duplicate plaque lifts are made on Schleicher and Schuell Optitran™ filters.

[0115] A probe is generated by PCR with primers Kid-366 and Kid-367, corresponding to nucleotides 397-420 of the AA050083 sequence. PCR reaction is set up as follows: 10 ul 10X PFU Buffer, 2.0 ul 10 mM dNTP mix, 72.1 ul H<sub>2</sub>O, 3.1 ul 13.2 uM Kid-367, 6.8 ul 5.88 uM Kid-366, 5.0 ul 0.1 ug/ul AA050083 DNA and 2.0 ul 2.5 Units/ul PFU (Stategene catalog #600154) are mixed. PCR is carried out in Perkin-Elmer Cetus DNA Thermal Cycler 480 with the following conditions: 25 cycles of 94C for 1 min, 53C for 1 min., 72C for 4 min. The product is purified by extraction with phenol, chloroform, isoamyl alcohol 50:49:1 followed by low-melt agarose gel electrophoresis and QiaexII purification of the excised fragment. This coding region probe is P<sup>32</sup> labeled by random priming (Feinberg and Vogelstein). The filters are hybridized overnight in 200 ml Plaque screen buffer ( ) containing 10% dextran sulphate, 100 ug/ml tRNA and 1.8 X 10<sup>8</sup> CPM of the mouse probe at 65C. They are washed twice with plaque screen buffer, twice with 2XSSC/1% SDS, twice with 1XSSC/1%SDS at 65C and exposed to film at -70C with an intensifying screen. Duplicate positives are plaque purified. Lambda miniprep DNA from the purified candidate plaques is digested with EcoR1, electrophoresed on a 1% agarose gel and Southern blotted. The Southern blot is hybridized with the mouse probe. Clone GJ128 , which has a 1.3 kb insert, hybridizes intensely to the mouse coding region probe. DNA sequence (partial human retL3 cDNA; SEQ ID NO:18) and deduced peptide sequence (partial human RetL3; SEQ ID NO:19) are obtained from this clone, confirming that it is the human homologue. This clone encodes most of the coding region, including the 3' end of the coding region.

[0116] The 1.3 kb insert from GJ128 is purified, labeled with P<sup>32</sup> and used to screen the Clontech human adult heart library in order to obtain a clone with the 5' end. No clones containing the 5' end are obtained in a screen of 2 X 10<sup>6</sup> plaques from this library. Northern analysis of human adult tissue mRNA blots (Clontech catalog #7760-1, 7759-1 and 7767-1) hybridized with the same probe, using protocols supplied by manufacturer, indicates that human RetL3 is expressed in human adult spinal cord, stomach, heart, pancreas, small intestine, colon, prostate and testis. A Clontech human adult spinal cord cDNA library (catalog #5001a) is screened with GJ128 insert. 3 independent clones are purified and the longest, GJ135 is sequenced. The sequence of the insert of GJ135 overlaps with the insert of GJ128, allowing the generation of a composite sequence of the full-length human retL3 cDNA (SEQ ID NO:20) and the determination of the full-length human RetL3 (SEQ ID NO:21). These sequences are also shown in Figure 10. Human RetL3 is 34.3% and 34.9% identical to human RetL1 and human RetL2, respectively. It has 76.8% identity with murine RetL3.

#### **THERAPEUTIC USES OF THE COMPOUNDS OF THE INVENTION**

[0117] Native and variant RetL's, anti-RetL antibodies, anti-Ret antibodies, and fusion proteins of Ret and of RetL's may have therapeutic utility in situations where it is desirable to block or to activate the Ret signaling pathway, to stimulate renal and/or neuronal cell growth or survival in disease situations where these cells are lost or damaged, or to suppress growth of or to kill undesirable cells such as tumor cells that express Ret or a RetL.

[0118] In general, compounds of the invention that bind to Ret, inducing dimerization and/or autophosphorylation of Ret, are useful for stimulating growth of or limiting damage to Ret-expressing tissues. The compounds of the invention are useful for stimulating renal tissue growth and/or survival, supporting renal function, and in minimizing damage to renal tissue after various insults. Particular conditions which may be beneficially treated with the compounds of the invention include acute renal failure, acute nephritis, chronic renal failure, nephrotic syndrome, renal tubule defects, kidney transplants, toxic injury, hypoxic injury, and trauma. Renal tubule defects include those of either hereditary or acquired nature, such as polycystic

renal disease, medullary cystic disease, and medullary sponge kidney. This list is not limited, and may include many other renal disorders (see, e.g., *Harrison's Principles of Internal Medicine*, 13th ed., 1994, which is herein incorporated by reference.)

**[0119]** In other applications, the genes and proteins of the invention may be used to treat conditions where neural growth and regeneration is desirable. This would include any conditions involving disorders of neural degeneration, such as Alzheimer's disease, Parkinson's, Huntington's, Tourette's, amyotrophic lateral sclerosis, as well as motor neuron disease, demyelinating diseases such as multiple sclerosis, bacterial diseases such as meningitis, abscess, or empyema, viral diseases such as HIV-associated myelopathy, prion diseases including Creutzfeldt-Jakob disease. Also included are disorders of damage to neural tissue, whether caused by neoplastic impingement, trauma, or cerebrovascular events such as hemorrhage or emboli. Diseases of the cranial nerves and of the spinal cord, including disorders involving traumatic, inflammatory, congenital or vascular etiologies, are specifically included, as are disorders affecting the autonomic nervous system. Also included are developmental neural disorders such as mental retardation, autism, fetal alcohol syndrome, Down's syndrome, and cerebral palsy. The compounds of the invention may also be used to treat syndromes involving the peripheral nervous system. These disorders include those caused by any of the factors previously listed, and specifically include Lyme disease, HIV-associated neuropathies, polymyositis, muscular dystrophy, and myasthenia gravis.

**[0120]** Anti-RetL antibodies and Ret fusion proteins of the invention, which specifically bind to the protein of rat RetL, partial human RetL1, full-length human RetL1, human RetL2, murine RetL3 or human RetL3, or fragments of these proteins, are useful in several methods. The compounds may be used therapeutically to inhibit or block Ret receptor signaling, such as for blocking growth of tumors which depend on activation of Ret signaling for growth. These agents may also be fused to detectable markers, such as fluoroscopically or radiographically opaque substances, and administered to a subject to allow imaging of tissues which express a RetL. The agents may also be bound to substances, such as horseradish peroxidase, which can be used as immunocytochemical stains to allow visualization of areas of RetL-positive cells on histological

sections. A specific antibody could be used alone in this manner, and sites where it is bound can be visualized in a sandwich assay using an anti-immunoglobulin antibody which is itself bound to a detectable marker. Specific antibodies to any RetL are also useful in immunoassays to quantify the substance for which a given antibody has specificity. Specific antibodies to a RetL may also be bound to solid supports, such as beads or dishes, and used to remove the ligand from a solution, either for use in purifying the protein or in clearing it from the solution. Each of these techniques is routine to those of skill in the immunological arts.

**[0121]** Other methods of the invention include modulating Ret-RetL signaling by contacting Ret with an anti-Ret monoclonal antibody. The effect of such a mAb-Ret contact can be to either block or to stimulate activation of the Ret signaling pathway, depending on the characteristics of the interaction of each particular mAb with Ret. Certain mAbs interact with Ret as agonists, with the agonist mAb-Ret binding triggering the dimerization and autophosphorylation of Ret. Other mAbs act as Ret antagonists. The interaction of Ret with an antagonist mAb prevents Ret signaling activation by other RetL=s, or by complexes comprising RetL's, which would otherwise activate the Ret signaling pathway.

**[0122]** A RetL and/or antibodies to Ret or to a Ret fusion protein can be used to allow imaging of tissues which express Ret, or in the immunohistological or preparative methods described above for antibodies to a RetL.

**[0123]** Fusion proteins encompassing a RetL and/or anti-Ret antibodies can be used to specifically target medical therapies against cancers and tumors which express Ret. Such tumors might include the several different tumor phenotypes which have been associated with mutations in Ret (N. Engl. J. Med. 335:943-951, 1996; Nature 367:319-320, 1996; Trends Gen. 12:138-144, 1996). Therapeutic interventions against neoplasias which express a RetL utilize fusion proteins which incorporate Ret and/or an anti-RetL antibody. The anti-Ret antibody or anti-RetL antibody may be effective by itself through antibody-dependent and complement-dependent cytotoxicity mediated by the Fc domain. Such hybrid ligands and antibodies can be made more effective as cancer therapeutics by using them as delivery vehicles for antineoplastic drugs, toxins and cytotoxic radionuclides, such as yttrium 90. Cytotoxic effector cells may be targeted



to tumor cells using heteroconjugate antibodies, where an antibody specific for either Ret or for a RetL expressed by a tumor is covalently coupled to an antibody directed against a surface protein on cytotoxic effector cells, such as NK cells or CTLs.

**[0124]** One example of an anti-Ret antibody or RetL therapy is to conjugate the toxic A chain of ricin or a modified full-length form of ricin (which can no longer bind cells) to a RetL or to an antibody directed against the Ret polypeptide expressed on the surface of malignant cells. In another embodiment, a toxin is conjugated to Ret or to an anti-RetL antibody to selectively target and kill RetL-positive cells, such as a tumor expressing a RetL. Such an approach has proved successful with blocked ricin conjugated to a monoclonal antibody against the CD19 antigen expressed on most neoplastic cells (Grossbard et al., Blood 79:576,1992). Other toxins are equally useful, as known to those of skill in the art. Such toxins include, but are not limited to, pseudomonas exotoxin, diphtheria toxin, and saporin. This approach should prove even more successful using a RetL or anti-Ret antibody, as contrasted to the known anti-CD19 antigen approach, because Ret is expressed in a very limited number of tissues.

**[0125]** The above approaches, using fusions of ricin or other toxins, are equally applicable to toxic conjugates of RetL or of an anti-Ret antibody; these are useful for selectively targeting and killing Ret-positive cells, such as tumor cells expressing Ret.

**[0126]** Another approach to such medical therapies is to use radioisotope labeled RetL or anti-Ret antibodies. Such radiolabeled compounds will preferentially target radioactivity to tumor sites in cells expressing Ret, sparing normal tissues. Depending on the radioisotope employed, the radiation emitted from a radiolabeled antibody bound to a tumor cell may also kill nearby malignant tumor cells that do not express Ret. A variety of radionuclides may be used. Isotopes that emit  $\beta$  particles (for example,  $^{131}\text{I}$ ) have been successful when employed with monoclonal antibodies against CD20 present on B-cell lymphomas (Kaminski et al., N. Engl. J. Med. 329:459, 1993; Press et al., N. Engl. J. Med. 329:1219, 1993). Radionuclides emitting  $\beta$  particles generate radioactive emissions that are tumoricidal over distances spanning several cell diameters, permitting the eradication of antigen negative cells and diminishing the consequences of nonhomogenous deposition of antibody or ligand in tumors.

[0127] Radionuclides emitting  $\alpha$  particles may also be employed. The low dose rate irradiation generated by radionuclide labeled RetL or anti-Ret antibodies may be more therapeutically effective than the instantaneous irradiation delivered externally in conventional radiation therapy. Low dose rate irradiation can induce apoptosis (programmed cell death) in certain cell lines (Macklis et al., Radiat. Res. 130:220, 1992; Maklis et al., Radiopharm. 5:339, 1992).

[0128] The compounds of the invention are administered in therapeutically-effective amounts, which means an amount of a compound which produces a medically desirable result or exerts an influence on the particular condition being treated.

[0129] The term "subject" used herein is taken to mean any mammal to which Ret ligand or gene may be administered. Subjects specifically intended for treatment with the method of the invention include humans, as well as nonhuman primates, sheep, horses, cattle, goats, pigs, dogs, cats, rabbits, guinea pigs, hamsters, gerbils, rats and mice, as well as the organs, tumors, and cells derived or originating from these hosts.

#### **Use of Compounds of the Invention in Gene Therapy**

[0130] The RetL genes of the invention are introduced into damaged tissue to stimulate production of a RetL by the transfected cells, to promote cell growth and/or survival of cells that express Ret.

[0131] In a specific embodiment of a gene therapy method a RetL gene may be introduced into a renal or neural target tissue of choice. A RetL would then be stably expressed and stimulate Ret receptor-positive cells to grow, divide, differentiate, and/or potentiate cell survival. Furthermore, RetL genes may be introduced into a target cell using a variety of well-known methods that use either viral or non-viral based strategies.

[0132] Non-viral methods include electroporation, membrane fusion with liposomes, high velocity bombardment with DNA-coated microprojectiles, incubation with calcium-phosphate-DNA precipitate, DEAE-dextran mediated transfection, and direct micro-injection into single cells. For instance, a RetL gene may be introduced into a cell by calcium phosphate

coprecipitation (Pillicer et al., *Science*, 209:1414-1422, 1980); mechanical microinjection and/or particle acceleration (Anderson et al., *Proc. Natl. Acad. Sci. USA*, 77:5399-5403, 1980); liposome based DNA transfer (e.g., LIPOFECTIN-mediated transfection- Fefgner et al., *Proc. Nat. Acad. Sci., USA*, 84:471-477, 1987; Gao and Huang, *Biochim. Biophys. Res. Comm.*, 179:280-285, 1991; DEAE Dextran-mediated transfection; electroporation (U.S. Patent 4,956,288); or polylysine-based methods in which DNA is conjugated to deliver DNA preferentially to liver hepatocytes (Wolff et al., *Science*, 247:465-468, 1990; Curiel et al., *Human Gene Therapy* 3:147-154, 1992).

**[0133]** Target cells may be transfected with the genes of the invention by direct gene transfer. See, e.g., Wolff et al., "Direct Gene Transfer Into Moose Muscle In Vivo", *Science* 247:1465-68, 1990. In many cases, vector-mediated transfection will be desirable. Any of the methods known in the art for the insertion of polynucleotide sequences into a vector may be used. See, for example, Sambrook et al., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1989) and Ausubel et al., *Current Protocols in Molecular Biology*, J. Wiley & Sons, NY (1992), both of which are incorporated herein by reference. Promoter activation may be tissue specific or inducible by a metabolic product or administered substance. Such promoters/enhancers include, but are not limited to, the native RetL promoter, the cytomegalovirus immediate-early promoter/enhancer (Karasuyama et al., *J. Exp. Med.*, 169:13, 1989); the human beta-actin promoter (Gunning et al., *Proc. Nat. Acad. Sci. USA*, 84:4831, 1987); the glucocorticoid-inducible promoter present in the mouse mammary tumor virus long terminal repeat (MMTV LTR) (Klessig et al., *Mol. Cell. Biol.*, 4:1354, 1984); the long terminal repeat sequences of Moloney murine leukemia virus (MuLV LTR) (Weiss et al., *RNA Tumor Viruses*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1985)); the SV40 early region promoter (Bernoist and Chambon, *Nature*, 290:304, 1981); the promoter of the Rous sarcoma virus (RSV) (Yamamoto et al., *Cell*, 22:787, 1980); the herpes simplex virus (HSV) thymidine kinase promoter (Wagner et al., *Proc. Nat. Acad. Sci. USA*, 78:1441, 1981); the adenovirus promoter (Yamada et al., *Proc. Nat. Acad. Sci. USA*, 82:3567, 1985).

**[0134]** The RetL genes may also be introduced by specific viral vectors for use in gene transfer systems which are now well established. See for example: Madzak et al., J. Gen. Virol., 73:1533-36, 1992 (papovavirus SV40); Berkner et al., Curr. Top. Microbiol. Immunol., 158:39-61, 1992 (adenovirus); Hofmann et al., Proc. Natl. Acad. Sci. 92:10099-10103, 1995 (baculovirus); Moss et al., Curr. Top. Microbiol. Immunol., 158:25-38, 1992 (vaccinia virus); Muzyczka, Curr. Top. Microbiol. Immunol., 158:97-123, 1992 ( adeno-associated virus); Margulskes, Curr. Top. Microbiol. Immunol., 158:67-93, 1992 (herpes simplex virus (HSV) and Epstein-Barr virus (EBV)); Miller, Curr. Top. Microbiol. Immunol., 158:1-24, 1992 (retrovirus); Brandyopadhyay et al., Mol. Cell. Biol., 4:749-754, 1984 (retrovirus); Miller et al., Nature, 357:455-450, 1992 (retrovirus); Anderson, Science, 256:808-813, 1992 (retrovirus), Current Protocols in Molecular Biology: Sections 9.10-9.14 (Ausubel et al., Eds.), Greene Publishing Associates, 1989, all of which are incorporated herein by reference.

**[0135]** Preferred vectors are DNA viruses that include adenoviruses (preferably Ad-2 or Ad-5 based vectors), baculovirus, herpes viruses (preferably herpes simplex virus based vectors), and parvoviruses (preferably "defective" or non-autonomous parvovirus based vectors, more preferably adeno-associated virus based vectors, most preferably AAV-2 based vectors). See, e.g., Ali et al., Gene Therapy 1:367-384, 1994; U.S. Patent 4,797,368 and 5,399,346 and discussion below.

**[0136]** The choice of a particular vector system for transferring, for instance, a RetL sequence will depend on a variety of factors. One important factor is the nature of the target cell population. Although retroviral vectors have been extensively studied and used in a number of gene therapy applications, they are generally unsuited for infecting cells that are not dividing but may be useful in cancer therapy since they only integrate and express their genes in replicating cells. They are useful for *ex vivo* approaches and are attractive in this regard due to their stable integration into the target cell genome.

**[0137]** Adenoviruses are eukaryotic DNA viruses that can be modified to efficiently deliver a therapeutic or reporter transgene to a variety of cell types. The general adenoviruses types 2 and 5 (Ad2 and Ad5, respectively), which cause respiratory disease in humans, are currently being

developed for gene therapy of Duchenne Muscular Dystrophy (DMD) and Cystic Fibrosis (CF). Both Ad2 and Ad5 belong to a subclass of adenovirus that are not associated with human malignancies. Adenovirus vectors are capable of providing extremely high levels of transgene delivery to virtually all cell types, regardless of the mitotic state. High titers ( $10^{13}$  plaque forming units/ml) of recombinant virus can be easily generated in 293 cells (an adenovirus-transformed, complementation human embryonic kidney cell line: ATCC CRL1573) and cryo-stored for extended periods without appreciable losses. The efficacy of this system in delivering a therapeutic transgene *in vivo* that complements a genetic imbalance has been demonstrated in animal models of various disorders. See Y. Watanabe, *Atherosclerosis*, 36:261-268, 1986; Tanzawa et al, *FEBS Letters*, 118(1):81-84, 1980; Golasten et al, *New Engl.J. Med.*, 309(11983):288-296, 1983; Ishibashi et al, *J. Clin. Invest.*, 92:883-893, 1993; and Ishibashi et al, *J. Clin. Invest.*, 93:1889-1893, 1994, all of which are incorporated herein by reference. Indeed, recombinant replication defective adenovirus encoding a cDNA for the cystic fibrosis transmembrane regulator (CFTR) has been approved for use in at least two human CF clinical trials. See, e.g., J. Wilson, *Nature*, 365:691-692 (Oct. 21, 1993). Further support of the safety of recombinant adenoviruses for gene therapy is the extensive experience of live adenovirus vaccines in human populations.

**[0138]** Human adenoviruses are comprised of a linear, approximately 36 kb double-stranded DNA genome, which is divided into 100 map units (m.u.), each of which is 360 bp in length. The DNA contains short inverted terminal repeats (ITR) at each end of the genome that are required for viral DNA replication. The gene products are organized into early (E1 through E4) and late (L1 through L5) regions, based on expression before or after the initiation of viral DNA synthesis. See, e.g., Horwitz, *Virology*, 2d edit., ed. B.N. Fields, Raven Press Ltd., New York (1990).

**[0139]** The first-generation recombinant, replication-deficient adenoviruses which have been developed for gene therapy of DMD and other inherited disorders contain deletions of the entire E1a and part of the E1b regions. This replication-defective virus is grown in 293 cells containing a functional adenovirus E1a gene which provides a transacting E1a protein. E1-deleted viruses

are capable of replicating and producing infectious virus in the 293 cells, which provide E1a and E1b region gene products in *trans*. The resulting virus is capable of infecting many cell types and can express the introduced gene (providing it carries its own promoter), but cannot replicate in a cell that does not carry the E1 region DNA unless the cell is infected at a very high multiplicity of infection. Adenoviruses have the advantage that they have a broad host range, can infect quiescent or terminally differentiated cells such as neurons, and appear essentially non-oncogenic. Adenoviruses do not appear to integrate in to the host genome. Because they exist extrachromasomally, the risk of insertional mutagenesis is greatly reduced. Ali et al., *supra*, at 373. Recombinant adenoviruses (rAdV) produce very high titers, the viral particles are moderately stable, expression levels are high, and a wide range of cells can be infected. Their natural host cells are airway epithelium, so they are useful for therapy of lung cancers.

[0140] Baculovirus-mediated transfer has several advantages. Baculoviral gene transfer can occur in replicating and nonreplicating cells, and can occur in renal cells, as well as in hepatocytes, neural cells, spleen, skin, and muscle. Baculovirus is non-replicating and nonpathogenic in mammalian cells. Humans lack pre-existing antibodies to recombinant baculovirus which could block infection. In addition, baculovirus is capable of incorporating and transducing very large DNA inserts.

[0141] Adeno-associated viruses (AAV) have also been employed as vectors for somatic gene therapy. AAV is a small, single-stranded (ss) DNA virus with a simple genomic organization (4.7 kb) that makes it an ideal substrate for genetic engineering. Two open reading frames encode a series of *rep* and *cap* polypeptides. *Rep* polypeptides (*rep78*, *rep68*, *rep 62* and *rep 40*) are involved in replication, rescue and integration of the AAV genome. The *cap* proteins (VP1, VP2 and VP3) form the virion capsid. Flanking the *rep* and *cap* open reading frames at the 5' and 3' ends are 145 bp inverted terminal repeats (ITRs), the first 125 bp of which are capable of forming Y- or T-shaped duplex structures. Of importance for the development of AAV vectors, the entire *rep* and *cap* domains can be excised and replaced with a therapeutic or reporter transgene. See B.J. Carter, in *Handbook of Parvoviruses*, ed., P. Tijsser, CRC Press, pp. 155-168

(1990). It has been shown that the ITRs represent the minimal sequence required for replication, rescue, packaging, and integration of the AAV genome.

[0142] The AAV life cycle is biphasic, composed of both latent and lytic episodes. During a latent infection, AAV virions enter a cell as an encapsulated ssDNA, and shortly thereafter are delivered to the nucleus where the AAV DNA stably integrates in to a host chromosome without the apparent need for host cell division. In the absence of a helper virus, the integrated AAV genome remains latent but capable of being activated and rescued. The lytic phase of the life cycle begins when a cell harboring an AAV provirus is challenged with a secondary infection by a herpesvirus or adenovirus which encodes helper functions that are recruited by AAV to aid in its excision from host chromatin (B.J. Carter, supra). The infecting parental ssDNA is expanded to duplex replicating form (RF) DNAs in a *rep* dependent manner. The rescued AAV genomes are packaged into preformed protein capsids (icosahedral symmetry approximately 20 nm in diameter) and released as infectious virions that have packaged either + or - ssDNA genomes following cell lysis.

[0143] Adeno-associated viruses (AAV) have significant potential in gene therapy. The viral particles are very stable and recombinant AAVs (rAAV) have "drug-like" characteristics in that rAAV can be purified by pelleting or by CsCl gradient banding. They are heat stable and can be lyophilized to a powder and rehydrated to full activity. Their DNA stably integrates into host chromosomes so expression is long-term. Their host range is broad and AAV causes no known disease so that the recombinant vectors are non-toxic.

[0144] Once introduced into a target cell, sequences of interest can be identified by conventional methods such as nucleic acid hybridization using probes comprising sequences that are homologous/complementary to the inserted gene sequences of the vector. In another approach, the sequence(s) may be identified by the presence or absence of a marker gene function (e.g, thymidine kinase activity, antibiotic resistance, and the like) caused by introduction of the expression vector into the target cell.

**Formulations and Administration**

**[0145]** The compounds of the invention may be administered in any manner which is medically acceptable. This may include injections, by parenteral routes such as intravenous, intravascular, intraarterial, subcutaneous, intramuscular, intratumor, intraperitoneal, intraventricular, intraepidural, or others as well as oral, nasal, ophthalmic, rectal, or topical. Sustained release administration is also specifically included in the invention, by such means as depot injections or erodible implants. Localized delivery is particularly contemplated, by such means as delivery via a catheter to one or more arteries, such as the renal artery or a vessel supplying a localized tumor.

**[0146]** The term "pharmaceutically acceptable carrier" means one or more organic or inorganic ingredients, natural or synthetic, with which the mutant proto-oncogene or mutant oncoprotein is combined to facilitate its application. A suitable carrier includes sterile saline although other aqueous and non-aqueous isotonic sterile solutions and sterile suspensions known to be pharmaceutically acceptable are known to those of ordinary skill in the art. In this regard, the term "carrier" encompasses liposomes and the HIV-1 tat protein (See Chen et al., Anal. Biochem. 227:168-175, 1995) as well as any plasmid and viral expression vectors. An "effective amount" refers to that amount which is capable of ameliorating or delaying progression of the diseased, degenerative or damaged condition. An effective amount can be determined on an individual basis and will be based, in part, on consideration of the symptoms to be treated and results sought. An effective amount can be determined by one of ordinary skill in the art employing such factors and using no more than routine experimentation.

**[0147]** The liposome system may be any variety of unilamellar vesicles, multilamellar vesicles, or stable plurilamellar vesicles, and may be prepared and administered according to methods well known to those of skill in the art, for example in accordance with the teachings of United States Patents 5,169,637, 4,762,915, 5,000,958 or 5,185,154. In addition, it may be desirable to express the novel polypeptides of this invention, as well as other selected polypeptides, as lipoproteins, in order to enhance their binding to liposomes. As an example, treatment of human acute renal failure with liposome-encapsulated RetL may be performed



*in vivo* by introducing a RetL into cells in need of such treatment using liposomes. The liposomes can be delivered via catheter to the renal artery. The recombinant RetL protein is purified, for example, from CHO cells by immunoaffinity chromatography or any other convenient method, then mixed with liposomes and incorporated into them at high efficiency. The encapsulated protein may be tested *in vitro* for any effect on stimulating cell growth.

**[0148]** This invention also contemplates that the novel polypeptide of this invention may be administered to an animal via liposome delivery system in order to enhance their stability and/or immunogenicity. Delivery of the novel polypeptides via liposomes may be particularly advantageous because the liposome may be internalized by phagocytic cells in the treated animal. Such cells, upon ingesting the liposomal membrane and subsequently present the polypeptides to the immune system in conjunction with other molecules required to elicit a strong immune response.

**[0149]** Any of the novel RetL polypeptides of this invention may be used in the form of a pharmaceutically acceptable salt. Suitable acids and bases which are capable of forming salts with the polypeptides of the present invention are well known to those of skill in the art, and include inorganic and organic acids and bases.

**[0150]** Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be obvious to one skilled in the art that certain changes and modifications may be practiced within the scope of the invention, as limited only by the scope of the appended claims.